APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1987, p. 189–192
0099-2240/87/010189-04$02.00/0

Vol. 53, No. 1

Isolation from Swine Feces of a Bacterium which Decarboxylates *p*-Hydroxyphenylacetic Acid to 4-Methylphenol (*p*-Cresol)†

LINDA A. WARD,[1]‡ KRISTEN A. JOHNSON,[1] ISADORE M. ROBINSON,[2] AND MELVIN T. YOKOYAMA[1]*

Department of Animal Science, Michigan State University, East Lansing, Michigan 48824-1225,[1] and National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa 50010[2]

Received 23 June 1986/Accepted 29 September 1986

An obligate anaerobe has been isolated from swine feces which decarboxylates *p*-hydroxyphenylacetic acid to 4-methylphenol (*p*-cresol). The bacterium was an ovoid rod, gram positive, nonsporeforming, and nonmotile. Lactate and acetate were major end products of glucose fermentation. Based on its characteristics, the bacterium is tentatively assigned to the genus *Lactobacillus*.

4-Methylphenol (*p*-cresol) is produced in the intestinal tract by the bacterial degradation of tyrosine (12). Two key reactions appear to be involved: transamination of L-tyrosine to *p*-hydroxyphenylacetic acid (pHPAA), followed by the decarboxylation of pHPAA to 4-methylphenol. The reactions may be carried out by either a single species or the interaction of two species. *Clostridium difficile* strains NCIB 10666 and ATCC 9689 produce 4-methylphenol from tyrosine (2, 4). However, other species, such as *Proteus vulgaris* (15) and several clostridial species (4), produce pHPAA from tyrosine but do not decarboxylate it to 4-methylphenol. There are also species which are capable of decarboxylating PHPAA to 4-methylphenol, but which do not produce 4-methylphenol directly from tyrosine (17).

The predominant volatile, phenolic metabolite excreted in the urine and feces of swine is 4-methylphenol (14, 18). 4-Methylphenol is a major contributor to the characteristic malodor associated with swine feces and housing facilities (6, 16), and studies (18) suggest that it may have a growth-depressing effect in young pigs. Based on radiolabeling studies with swine feces, 4-methylphenol is produced from tyrosine via pHPAA, and the process involves more than one species (14). The present study describes the isolation and characteristics of a bacterium from swine feces which produces 4-methylphenol by the decarboxylation of pHPAA.

Newly weaned pigs (4.5 to 6.8 kg in body weight) were fed a ground, shelled corn and soybean meal diet, supplemented with a commercial antibiotic premix (ASP-250; containing chlortetracycline, sulfamethazine, and penicillin). Freshly voided feces were collected in presterilized plastic cups. One gram of feces was inoculated under CO_2 into a selective basal PYG medium and incubated at 39°C. Anaerobic culturing methods used were essentially as described by Hungate (8), as modified by Bryant (1). The basal PYG medium used to select for the 4-methylphenol-producing bacteria consisted of (percent): peptone, 0.5; yeast extract, 1.0; glucose, 0.5; mineral solution no. 1, 8.3; mineral solution no. 2, 8.3; hemin-menadione solution, 1.0; resazurin solution, 0.1; 4-methylphenol, 0.2; sodium carbonate solution, 5.0; and cysteine-sulfide solution, 2.0. The composition of mineral solutions 1 and 2 was as previously described (17). Hemin-menadione and resazurin solutions were prepared by the Virginia Polytechnic Institute *Anaerobe Laboratory Manual* procedures (7). Sodium carbonate and cysteine-sulfide were prepared as previously described (17) and added aseptically to the medium after autoclaving. The medium was autoclaved at 15 lb/in^2 for 20 min and dispensed in 18- by 150-mm tubes. 4-Methylphenol was added to the basal PYG medium on the premise that the 4-methylphenol-producing bacteria would be more tolerant of its bactericidal effects as was previously demonstrated at 0.2% for *C. difficile* (S. Hafiz, Ph.D. thesis, University of Leeds, Leeds, U.K., 1974). At this concentration, 4-methylphenol was effective in facilitating an isolation by eliminating the more sensitive species and selecting for the 4-methylphenol-producing species. All attempts to isolate 4-methylphenol-producing bacteria were made on subcultures of the mixed fecal population maintained on the 0.2% 4-methylphenol-containing medium with daily transfers. In screening for isolates, pHPAA was added at 6.6 mM to the selective medium, and the 4-methylphenol was deleted. The pHPAA was dissolved in 1 ml of 1 N NaOH prior to its addition to the medium.

The anaerobic roll tube technique of Hungate (9) was used to isolate the 4-methylphenol-producing bacteria. Cultures were serially diluted 10-fold in anaerobic dilution solution, containing mineral solutions 1 and 2, hemin, menadione, and resazurin as described for the basal PYG medium. A 1-ml portion of the 10^{-6} to 10^{-10} dilutions was inoculated into triplicate roll tubes of the selective medium containing 2% agar and incubated at 39°C. Well-isolated colonies formed after 3 to 5 days were picked under CO_2 and transferred to the basal PYG medium containing 6.6 mM pHPAA. After 24 h of incubation at 39°C, the isolates were subcultured into fresh basal PYG medium, and the spent cultures were analyzed for 4-methylphenol production by gas-liquid chromatography (17). This procedure was repeated on 4-methylphenol-positive cultures until all of the colonies in subsequent roll tubes were similar in morphological type and microscopic examination showed that the cultures were pure in three consecutive subcultures.

A 4-methylphenol-producing isolate was obtained from a 10^{-6} dilution roll tube and was found to be a gram-positive, nonmotile, and nonsporeforming ovoid rod (0.8 by 0.6 μm). It occurred predominantly as a diploid (Fig. 1), but formed short chains of 4 to 12 cells. In older cultures, the bacterium formed a phlegmlike sediment which adhered to the sides and bottom of the tube. Surface colonies in roll tubes were

* Corresponding author.

† Michigan Agricultural Experiment Station Journal Article no. 11962.

‡ Present address: Medical College of Ohio at Toledo, Toledo, OH 43699.

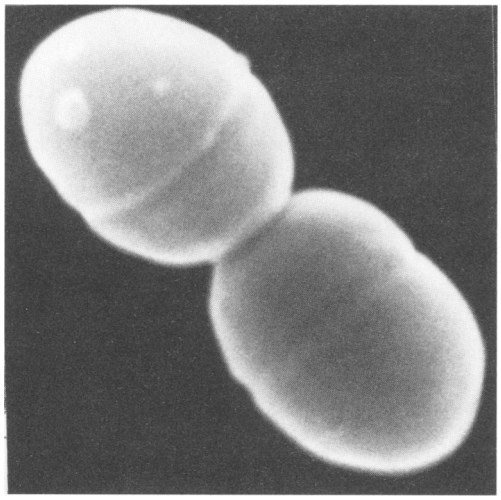

FIG. 1. Scanning electron photomicrograph of the *p*-cresol-producing bacterium isolated from swine feces. The bacterium was grown in basal PYG medium for 24 h at 39°C. Magnification, ×30,000.

white to opaque in color and round with entire edges. Deep colonies embedded in the agar were lenticular in shape and about 1 mm in diameter after 1 week of incubation. Efforts to isolate other 4-methylphenol-producing bacteria, either directly from serially diluted feces or by the selective enrichment procedure, were unsuccessful. The 4-methylphenol-producing isolate was maintained in 2% agar slants of a modified (17) MRS lactobacillus medium (3) held at 4°C, with monthly transfers.

The occurrence of the 4-methylphenol-producing bacterium in swine feces was estimated by the most-probable-number technique (5). Freshly voided feces from eight weanling pigs were serially diluted 10-fold in anaerobic dilution solution. A 1-ml amount of the dilutions was then inoculated into triplicate tubes of the basal PYG medium containing 13.1 mM pHPAA, but no glucose. Incubation was for 48 h at 39°C. Most probable number was estimated from the 4-methylphenol concentrations in the dilution tubes compared with control tubes with no pHPAA added. The occurrence of the bacterium was 0.05×10^7 per g of feces.

The media for the nutritional and biochemical characterization tests were prepared as described in the Virginia Polytechnic Institute *Anaerobe Laboratory Manual* (7). The bacterium fermented and grew on a wide variety of carbohydrate substrates, including glucose, fructose, maltose, mannose, rhamnose, salicin, sucrose, and xylose. It would not ferment adonitol, amygdalin, arabinose, cellobiose, erythitol, glycogen, myo-inositol, inulin, lactose, D-mannitol, D-melibiose, melizitose, D-(+)-raffinose, D-ribose, D-sorbitol, trehalose, galactose, and starch. Tests were negative for catalase, gelatin liquefaction, ammonia production, hemolysis, indole, and litmus milk. Nitrate reduction and chopped-meat broth tests were weakly positive. Tween 80 stimulated the growth of the bacterium and was added at 0.1% to all subsequent medium preparations. Neither clarified swine fecal extract nor clarified rumen fluid at 20% was stimulatory for growth of the bacterium. Growth occurred at 39°C, weakly at 30°C, and not at all at 45°C. The bacterium failed to grow if the resazurin was oxidized. Optimum growth was obtained when the pH was 5.0 to 5.5. Major end products of glucose fermentation were lactate and acetate at 64.8 and 35.2 mol%, respectively. Trace amounts of ethanol and formate were also detected, with no gas production.

4-Methylphenol was not produced when the bacterium was grown in the basal PYG medium. However, when the medium contained 6.6 mM pHPAA, a strong odor characteristic of 4-methylphenol was detected in cultures after 24 h. Analysis of the ether extract of cultures by thin-layer chromatography (13) yielded a metabolite with the same R_f value (0.71) and color reaction (pink) as 4-methylphenol. A peak with the same relative retention time as 4-methylphenol was also detected with gas-liquid chromatography. When challenged with increasing amounts of pHPAA, the bacterium produced up to 37 mM 4-methylphenol with a yield of 76 mol% in 24 h.

The specificity of the decarboxylation reaction was tested with various substrates, and metabolites produced were identified by gas-liquid and thin-layer chromatography (13, 17). 4-Methylphenol was not produced from L-tyrosine by the bacterium, and neither phenylalanine nor tryptophan was degraded. Although pHPAA was decarboxylated to 4-methylphenol, neither *m*-hydroxyphenylacetic acid nor *o*-hydroxyphenylacetic acid was decarboxylated to its corresponding isomer. This suggested that a hydroxyl group in the para position was essential for the decarboxylation reaction. If the para hydroxyl group was substituted with either a fluoro or a nitro group (i.e., *p*-fluorophenylacetic acid, *p*-nitrophenylacetic acid, *o*-nitrophenylactic acid), or if the para hydroxyl group was removed (i.e., phenylacetic acid), no decarboxylation occurred. The bacterium also would not decarboxylate indoleacetic acid to 3-methyl-

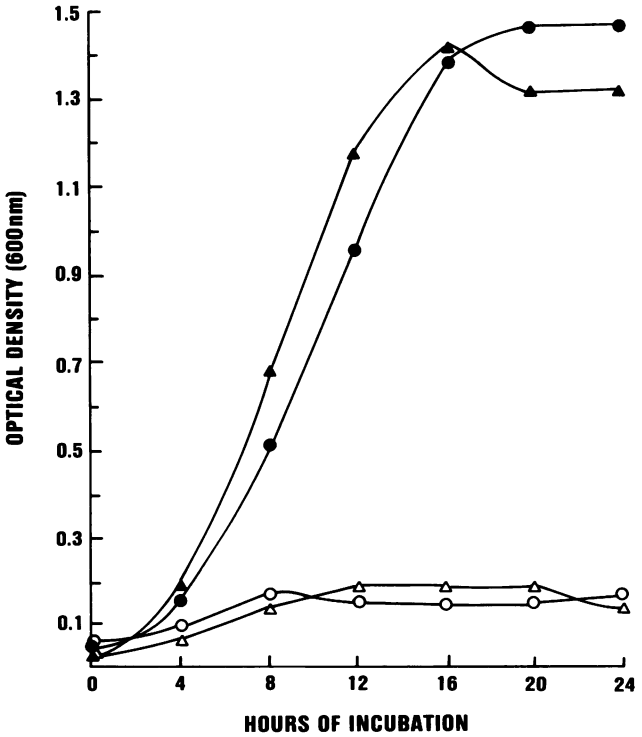

FIG. 2. Effect of pHPAA on growth of the *p*-cresol-producing bacterium. The bacterium was inoculated into basal PYG medium under the following conditions: no pHPAA (●); pHPAA, 21.9 mM (▲); no pHPAA, no glucose (○); pHPAA, 21.9 mM, no glucose (△). Incubation was for 24 h at 39°C. Values are the means of triplicate cultures.

indole. Of the compounds examined, 3,4-dihydroxyphenylacetic acid was the only other compound decarboxylated. Analysis by thin-layer chromatography showed that the bacterium produced methylcatechol from 3,4-dihydroxyphenylacetic acid. Neither 2,5-dihydroxyphenylacetic acid nor 3,4-dimethoxyphenylacetic acid was decarboxylated. It appeared from these results that a hydroxyl group in the 3 position of the phenolic ring did not hinder the decarboxylation, but hydroxyl groups in the 2 and 5 positions and methoxyl groups in the 3 and 4 positions would not substitute for a para hydroxyl group.

pHPAA was not essential for growth of the bacterium and did not serve as the sole source of energy when substituted for glucose in the medium (Fig. 2). Although the data suggested that pHPAA might be stimulatory for growth when added to the basal PYG medium, an increased growth rate was not always observed. What was consistently demonstrated with the addition of pHPAA was a decrease in the optical density of cultures during early stationary phase (Fig. 2). The 4-methylphenol concentrations were quite high by 16 h of incubation, and the decrease in optical density could be explained by a toxic effect on the bacterium.

When inoculated into medium containing a low concentration of pHPAA (1.1 mM), but no glucose, the bacterium produced 4-methylphenol in the absence of growth (Fig. 3). 4-Methylphenol was detected in resting cultures within 4 h after inoculation, and its concentration was observed to increase until a plateau was reached after 8 h. About 90% of the added pHPAA was decarboxylated to 4-methylphenol by resting cultures. In contrast, when glucose was added to the medium, a noticeable lag was observed in 4-methylphenol production, with virtually no *p*-cresol detected by 4 h of incubation. However, as the growth of the bacterium ensued, the concentration of *p*-cresol rapidly increased until 8 h of incubation and then plateaued out to 24 h. About 86% of the added pHPAA was decarboxylated to 4-methylphenol by growing cultures.

The physiological significance of 4-methylphenol production to the bacterium is unclear. The free-energy change of the decarboxylation reaction is too low to support growth, but the high decarboxylase activity observed when pHPAA is provided suggests that the reaction is metabolically important to the bacterium. The site of intestinal colonization of the bacterium, based on the 4-methylphenol concentration of intestinal contents, is reported to be the cecum and colon; however, some 4-methylphenol is also detected in the stomach and small intestine (19).

Based on its physiological, biochemical, and metabolic characteristics, the 4-methylphenol-producing bacterium appears to belong in the genus *Lactobacillus*. Morphologically, the bacterium resembles the genus *Eubacterium*, but there is no gas production, and it does not produce any ammonia, propionate, or butyrate. The high lactate/acetate molar ratio of the isolate is also inconsistent with *Eubacterium*. The bacterium does not resemble the genus *Streptococcus* or *Bifidobacterium* in terms of morphology, carbohydrates fermented, and end products produced. Several *Lactobacillus* species (*L. acidophilus*, *L. leichmanii*, *L. delbrueckii*, *L. plantarum*, *L. brevis*, *L. minutis*, and *L. fermentus*) have been isolated from the intestinal tract of swine (10, 11). The present isolate, however, does not resemble any of these species, and there is no report that they will produce 4-methylphenol. The 4-methylphenol-producing isolate may be a new species of *Lactobacillus*, but a formal designation should await the isolation of other strains from the intestinal tract.

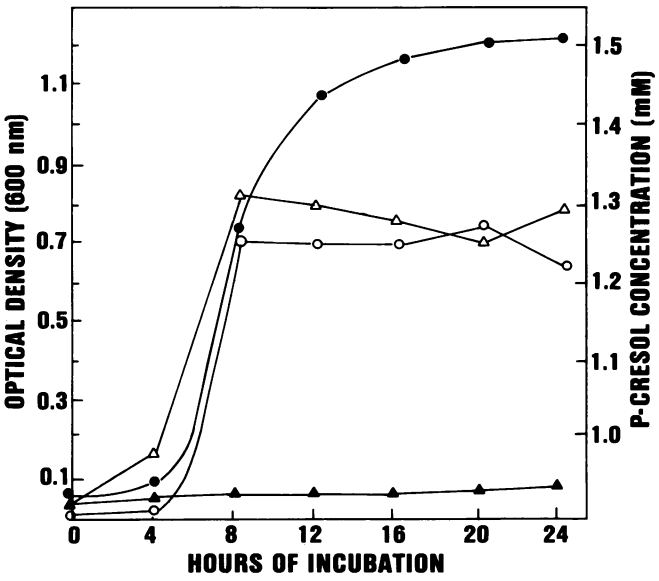

FIG. 3. Time sequence curves of growth and *p*-cresol production by the bacterium in the presence of a low pHPAA concentration (1.1 mM), with and without the addition of glucose. The bacterium was inoculated into 30 ml of the basal PYG medium, and 1 ml of culture was removed under CO_2 at 4-h intervals for analysis. Symbols: growth curve with no glucose added (▲); growth curve with glucose added (●); *p*-cresol concentration with no glucose added (△); *p*-cresol concentration with glucose added (○). Incubation was for 24 h at 39°C.

This research was partially supported by Michigan Agricultural Experiment Station project 1244, U.S. Department of Agriculture Science and Education Administration grant 59-2261-1-2-031-0, and the National Pork Producers Council, Des Moines, Iowa.

We thank E. R. Miller and I. G. Lumanta for assistance with the animals and S. L. Flegler for the scanning electron microscopy.

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